IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Before the Board of Patent Appeals and Interferences

In re Patent Application of

MAERTENS et al.

Serial No. 09/973,025

Filed: October 10, 2001

Title: PURIFIED HEPATITIS C VIRUS ENVELOPE PROTEINS FOR DIAGNOSTIC AND THERAPEUTIC USE

Atty Dkt. 2551-108
C# M#
TC/A.U.: 1648
Examiner: Li
Date: November 10, 2004

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

☐ **Correspondence Address Indication Form Attached.**

☐	**NOTICE OF APPEAL** Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the Examiner twice/finally rejecting ($340.00) applicant's claim(s).	$	
☒	An appeal **BRIEF** is attached in the pending appeal of the above-identified application ($ 340.00)	$	340.00
☐	Credit for fees paid in prior appeal without decision on merits	-$ (	)
☐	A reply brief is attached in triplicate under Rule 41.41		**(no fee)**
☐	Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) (**$110.00/1 month**; $430.00/2 months; **$980.00/3 months**; $1530.00/4 months)	$	980.00
	SUBTOTAL	$	1320.00
☐	Applicant claims "Small entity" status, enter ½ of subtotal and subtract ☐ "Small entity" statement attached.	-$(	)
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Any future submission requiring an extension of time is hereby stated to include a petition for such time extension. The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140.** A duplicate copy of this sheet is attached.

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Signature: _____

cp 1648 IFW AF

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APPEAL BRIEF

Sir:

Applicant hereby appeals the file rejection of claims 100-104, 107-112 and 116-117, in the Office Action dated December 17, 2003, as supplemented by the comments in the Advisory Actions of June 16, 2004 and October 20, 2004, and submits the present Appeal Brief pursuant to 37 CFR § 41.37.

11/12/2004 KBETEMA1 00000029 09973025
01 FC:1402 340.00 OP

11/12/2004 KBETEMA1 00000029 09973025
02 FC:1253 980.00 OP

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(1) REAL PARTY IN INTEREST

The real party in interest is Innogenetics N.V., Industriepark Zwijnaarde 7 Box 4, B-9052 Ghent, Belgium, by way of an Assignment from the applicants, recorded in the U.S. Patent and Trademark Office on March 11, 1996, at Reel 8014, Frames 0961-0962.

(2) RELATED APPEALS AND INTERFERENCES

The appellant, the appellant's legal representative, and the assignee are not aware of any related prior or pending appeals or interferences or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(3) STATUS OF THE CLAIMS

Claims 100-118 are pending. Claims 100-104, 107-112 and 116-117 have been finally rejected.

Claims 105, 106, 113-115 and 118 are objected to. Specifically, claims 106, 113-115 and 118 are not in condition for allowance because they depend on rejected claim 100. See, page 2 of the Advisory Action dated October 20, 2004. Claim 105 is not "in the condition for allowance because the scope of the claim 115 reads more broad then the independent claim 100." Id. There is no stated rejection of claims 105, 106, 113-115 or 118 in the Advisory Action dated October 20, 2004.

Claims 100-104, 107-112 and 116-117 have been finally rejected and are the subject of this appeal.

Originally-filed claims 1-48 were canceled, without prejudice, and new claims 49-99 added in a Preliminary Amendment filed October 10, 2001. Claims 49-99 were canceled, without prejudice, and new claims 100-118 added by way of an Amendment filed September 9, 2003. Claims 110, 114 and 116 were amended March 17, 2004 in an Amendment after final rejection which was entered. See, page 2 of the Advisory Action dated June 16, 2004. An amendment to rewrite claim 114 as an independent claim was presented in a Second Amendment Under Rule 116 filed August 17, 2004. The Second Amendment Under Rule 116 filed August 17, 2004 was not entered however the Examiner indicated that "amended claim(s) 114 would

be allowable if submitted in a separate, timely filed Amendment canceling the non-allowable claim(s)." See, page 1 of the advisory Action dated October 20, 2004.

The Examiner's refusal to enter the Second Amendment Under Rule 116, which the Examiner has admitted would place claim 114 in allowable form, is submitted to be contrary to 37 CFR § 1.116(b) as the amendment of claim 114 presents claim 114 in a better (i.e., allowable) form.

A copy of all the rejected claims 100-104, 107-112, and 116-117, i.e., the claims involved in the appeal, is attached as a Claims Appendix, pursuant to Rule 41.37(c)(1)(viii).

(4) STATUS OF THE AMENDMENTS

The Amendment filed March 17, 2004 has been entered. See, page 2 of the Advisory Action dated June 16, 2004. A Second Amendment Under Rule 116 filed August 17 2004 has not been entered. See, Advisory Action dated October 20, 2004.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Claim 100 is the only pending rejected independent claim. Claim 100 provides an isolated E2 specific monoclonal antibody which binds to at least one region within a domain spanning amino acids 416-650 or 655-809 of the hepatitis C polyprotein. Support for independent claim 100 may be found, for example, in the disclosure of pages 22-23 and 67. The appellants note that the E2 protein is defined in the present application as spanning amino acids 384-809, which is further defined by the regions of Table 3, on page 67 of the specification.

Support for the details of rejected, dependent claims 101 and 117 may be found, for example, spanning pages 22-23 of the specification.

Support for the details of rejected, dependent claim 102 may be found, for example, in the passage spanning lines 2-7 on page 12, of the specification.

Support for the details of rejected, dependent claim 103 may be found, for example, in the passage spanning lines 2-12 of page 19 of the specification.

Support for the details of rejected, dependent claim 104 may be found, for example, in the paragraph spanning pages 18-19 of the specification.

Support for the details of rejected, dependent claim 107 may be found, for example, in the paragraph spanning pages 22-23 of the specification.

Support for the details of rejected, dependent claim 108 may be found, for example, on page 8, lines 1-10 of the specification.

Support for the details of rejected, dependent claim 109 may be found, for example, on page 8, lines 1-10 of the specification.

Support for the details of rejected, dependent claim 110, may be found, for example, on page 8, line 10 of the specification.

Support for the details of rejected, dependent claim 111, may be found, for example, on page 8, line 10 of the specification.

Support for the details of rejected, dependent claim 112, may be found, for example, on page 8, line 11 of the specification.

Support for the details of rejected, dependent claim 116 may be found on pages 56-57 of the specification and generally throughout the specification wherein antibodies of the invention may be used in competition.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following ground of rejection is presented for review:

Whether the invention of claims 100-104, 107-112 and 116-117, is anticipated, under 35 USC § 102(a), by Mehta et al (U.S. Patent No. 5,308,750) .

(7) ARGUMENT

The invention of independent claim 100, and dependent claims 102-104, 107-112 and 116-117, are patentable over Mehta et al (U.S. Patent No. 5,308,750), which fails to provide each and every aspect of the presently claimed invention. The Section 102 rejection of claims 100-104, 107-112 and 116-117 should be reversed. Consideration of the following in this regard is requested.

Anticipation requires a teaching of each and every aspect of the claimed invention to be found in a prior art reference.

The presently claimed invention provides an isolated E2 specific monoclonal antibody which specifically binds to at least one region within a domain spanning amino acids 416-650 or 655-809 of the hepatitis C virus polyprotein.

Mehta et al describe, in Table 2 of the patent (column 16) describes monoclonal antibodies H13C113 and H23C163, and that SEQ ID NO:3 (aa 643-663) and SEQ ID NO:4 (aa 666-683) of Mehta et al have been separately used for epitope mapping of these antibodies. Mehta et al conclude that antibodies H13C113 and H23C163 of Mehta et al specifically react with SEQ ID NO:10 of Mehta et al (aa 649-655). See, Figures 3 and 4 and column 4, lines 19-26 of Mehta et al. Mehta et al therefore discloses, at best, two antibodies (i.e., H13C113 and H23C163), which bind to epitope(s) within the region of amino acids 649-655.

The Board will appreciate that the region of binding of the antibodies described by Mehta et al overlaps with the domain of amino acids 416-650 of independent claim 100 by two (2) amino acids (i.e., amino acids 649 and 650).

Mehta et al fails to disclose, literally or inherently, an antibody which binds to at least one region within a domain spanning amino acids 416-650 or 655-809 of the hepatitis C virus polyprotein as recited in independent claim 100 and interpreted by the Examiner.

The appellants understand the Examiner to have interpreted the rejected independent claim 100 as reading on "any monoclonal antibody that binds to a region that may be as short as 5 to 10 amino acids in the two claimed domains." See, ¶4 page 3 of the Advisory Action dated October 20, 2004.

The appellants understand the Examiner's interpretation of rejected independent claim 100 to require the binding of the claimed monoclonal antibody to at least one region, which may be as short as 5 to 10 amino acids, within the recited domains spanning amino acids 416-650 or 655-809 of the hepatitis C virus polyprotein.

By the Examiner's interpretation of rejected independent claim 100, the antibodies of Mehta et al which, at best, bind in a region of a two (2) amino acid overlap with the claimed region of amino acids 416-650, fail to fall within the scope of the rejected claim 100 and the Section 102 rejection of claims 100-104, 107-112 and 116-117 over Mehta et al should be reversed.

The Examiner has further stated the following, as the apparent basis for the rejection:

> "It is well known in the art that a monoclonal antibody is able to bind as short as 5 to 20 amino acids of an immunogenic epitop [sic] in an immunogenic polypeptide, and sometime [sic], a single amino acid residue contributes the majority of the binding interaction between the antibody and antigen. For example, Petit et al. (J. Biol. Chemistr. 2003, Vol. 278, pp. 45 [sic], pp 44385-44392) teach that the monoclonal antibody D32.10 binds several epitopes from 8 to 15 amino acid residues in the region of amino acid residues 607-627 of HCV E2 (See Table VI on page 44389) and E1 (See Table 1 and Fig. 2 on page 44388)." See, page 3 of the Advisory Action dated October 20, 2004 (emphasis added).

The Examiner's quoted comments appear to be inconsistent with the Examiner's interpretation of independent claim 100 which, as noted above, requires binding to a region that may be as short as 5 to 10 amino acids "in the claimed domains." See, ¶4 on page 3 of the Advisory Action. More importantly, the Examiner's apparent suggestion that a single amino acid would be sufficient to bind a monoclonal antibody is contrary to generally accepted textbook knowledge of peptide binding properties of an antibody. See also, page 10, lines 22-25 of the present application.

The Examiner's reliance on, and the relevance of, the newly cited Petit et al is not understood. Petit et al relates to epitope mapping of an antibody, D32.10, which binds to both E1 and E2. As indicated by the Examiner, the cysteine residues located at positions 306, 494 and 620 were amino acids which were found to react

strongly with antibody D32.10. The Examiner concludes from this that Petit suggests "that one single monoclonal antibody can bind to several epitopes of a polypeptide." See, ¶5 of page 3 of the Advisory Action dated October 20, 2004.

The appellants do not disagree with the Examiner's statement that a monoclonal antibody may be able to bind to several epitopes of a polypeptide. In fact, Petit et al indicate that D32.10 binds to amino acids in sequences 292-306, 480-494 and 608-622, other than Cys^{306}, Cys^{494} and Cys^{620}, equally but more weakly than the noted cysteines.

Mehta et al suggests that the antibodies specifically relied upon by the Examiner bind in a region spanning amino acids 649-655. Mehta et al. do not teach or suggest that their antibodies H13C113 and H23C163 bind to several epitopes of the HCV polypeptide.

Moreover, the substitution of the cysteines is at positions 306, 494 and 620 of Petit et al.'s D32.10 with Ala did not lead to a lack antibody binding (see Figure 3 in Petit et al.). One of ordinary skill will appreciate from this that the noted cysteines alone are not sufficient to form an epitope, as suggested by the Examiner ("a single amino acid residue contributes the majority of binding interaction between the antibody and antigen").

Further, Mehta et al. appear to indicate in their Figures 3-4 that several contiguous amino acids of the HCV polypeptide are involved in the binding of their antibodies H13C113 and H23C163.

The Board is requested to appreciate that Petit et al. themselves state in the last sentence of the section "Immunoreactivity of Selected Phage-displayed E1 and E2 sequences with mAb D32.10" the following: "... it is difficult to discriminate between E2(480-494) and E2 (613-621) because these two regions contain the same motif (WHYP)...". Notably this "WHYP" motif is very close to Cys494 in peptide E2 (480-494) and is immediately adjacent to Cys620 in peptide E2 (608-622). Thus, Petit et al themselves admit that for the two E2 peptides Cys494 alone and Cys620 alone are not sufficient and indicate that these two peptides are in fact mimotopes of each other due to the common "WHYP" motif. Finally, the appellants note that none of the selected phage clones (selected for binding mAb D32.10) carries a peptide sequence (mimotope sequences to E1 or E2) comprising the allegedly very important Cys residues. In fact, there are no Cys residues at all in these peptides (see Tables I and II in Petit et al). This only adds to the fact that a Cys alone, or a single amino acid as suggested by the Examiner, is not sufficient and moreover not required to bind mAb D32.10.

The claims are submitted to be patentable over the cited Mehta et al patent and Reversal of the Final Rejection is requested.

Respectfully submitted,

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(8) CLAIMS APPENDIX

100. An isolated E2 specific monoclonal antibody, said antibody specifically binding to at least one region within a domain spanning amino acids 416-650 or 655-809 of the hepatitis C virus polyprotein.

101. The monoclonal antibody according to claim 100 or 113 which has been produced from a mammal immunized with a composition comprising purified recombinant HCV single or specific oligomeric recombinant E2 envelope proteins.

102. The monoclonal antibody according to claim 101 wherein said recombinant HCV E2 envelope proteins are produced by a recombinant mammalian cell.

103. The monoclonal antibody of claim 102 wherein said mammalian cell is infected with recombinant vaccinia virus carrying DNA for expressing said HCV E2 envelope proteins.

104 The monoclonal antibody according to claim 101 wherein said recombinant HCV E2 envelope proteins are produced by a recombinant yeast cell.

107. An isolated antibody of claim 101 which is an E2 specific monoclonal antibody.

108. The isolated protein of claim 101 wherein said E2 protein is at least 90% pure.

109. The isolated protein of claim 101 wherein said E2 protein is at least 95% pure.

110. The isolated protein of claim 101 wherein said E2 protein is at least 97% pure.

111. The isolated antibody of claim 101 wherein said E2 protein is at least 97% pure.

112. The isolated antibody of claim 101 wherein said E2 protein is at least 99% pure.

116. An isolated monoclonal antibody which competes for binding to an E2 protein with an E2 specific monoclonal antibody of claim 114.

117. The monoclonal antibody according to claim 100 or 113 which has been produced from a mammal immunized with a composition comprising at least one purified recombinant HCV single or specific oligomeric recombinant E2 envelope protein.

(9) EVIDENCE APPENDIX

Attached:

(a) Petit et al. (JBC, vol. 278, No. 45, Nov. 7, pp. 44385-44392 (2003).

Entered in record by Examiner with October 20, 2004 Advisory Action and attached PTO 892 Form.

(b) U.S. Patent No. 5,308,750.

Entered in record by Examiner with PTO 1449 Form signed by Examiner on "4/9/03" and returned to applicants with June 16, 2004 Advisory Action.

(9) RELATED PROCEEDINGS APPENDIX

Attached:

NONE

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 45, Issue of November 7, pp. 44385–44392, 2003
Printed in U.S.A.

Mapping of a Conformational Epitope Shared between E1 and E2 on the Serum-derived Human Hepatitis C Virus Envelope*

Received for publication, April 17, 2003, and in revised form, July 23, 2003
Published, JBC Papers in Press, July 24, 2003, DOI 10.1074/jbc.M304047200

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Monoclonal antibody D32.10 produced by immunizing mice with a hepatitis C virus (HCV)-enriched pellet obtained from plasmapheresis of a chronically HCV1b-infected patient binds HCV particles derived from serum of different HCV1a- and HCV1b-infected patients. Moreover, this monoclonal has been shown to recognize both HCV envelope proteins E1 and E2. In an attempt to provide novel insight into the membrane topology of HCV envelope glycoproteins E1 and E2, we localized the epitope recognized by D32.10 on the E1 and/or E2 sequence using Ph.D.-12™ phage display peptide library technology. Mimotopes selected from the phage display dodecapeptide library by D32.10 shared partial similarities with ^{297}RHWTTQGCNC^{306} of the HCV E1 glycoprotein and with both ^{613}YRLWHYPCT^{621} and ^{480}PDQRPYCWHYPPKPC^{494} of the HCV E2 glycoprotein. Immunoreactivity of D32.10 with overlapping peptides corresponding to these three HCV regions confirmed these localizations and suggested that the three regions identified are likely closely juxtaposed on the surface of serum-derived particles as predicted by the secondary model structure of HCV E2 derived from the tick-borne encephalitis virus E protein. This assertion was supported by the detection of specific antibodies directed against these three E1E2 regions in sera from HCV-infected patients.

Infection with hepatitis C virus (HCV)[1] represents an important public health problem worldwide because it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). HCV is an enveloped positive single-stranded RNA virus that is a member of the Flaviviridae family (2). It is now classified within a third genus, designated *Hepacivirus* (3). Its genomic organization consists of one large translational open reading frame encoding a polyprotein of ~3000 amino acids, which is bracketed by 5′- and 3′-noncoding regions (2). The HCV polyprotein is processed into functional proteins by host and viral proteases (4). The three structural proteins, *viz.* core and envelope glycoproteins E1 and E2, are located within the N terminus of the polyprotein, whereas the nonstructural proteins reside within the C-terminal part (5). Glycoproteins E1 and E2 are believed to be type 1 integral transmembrane proteins, with C-terminal hydrophobic anchor domains. Until now, structure-function analyses of HCV gene products have been carried out using artificial cell expression systems in which E1 and E2 form two kinds of complexes *in vitro* (6–9): high molecular mass aggregates that contain intermolecular covalent bonds and native complexes in which E1 and E2 associate by noncovalent interactions. The contribution of either complex to the structure of the proteins on viral particles is unknown; however, the latest complexes are thought to correspond to the native association of E1 and E2. The lack of an efficient tissue culture system for propagating the virus and low levels of HCV particles in plasma samples are the main factors responsible for slow progress in this research area.

In an attempt to obtain information on the exact nature of the interaction between E1 and E2, we recently produced murine monoclonal antibodies directed against E1E2 complexes expressed on native HCV particles isolated from serum of chronically infected patients.[2] In this study, the new monoclonal antibody (mAb) D32.10, which specifically recognizes serum-derived HCV particles and both HCV glycoproteins E1 and E2, was obtained. The fine epitope localization of mAb D32.10 was established using phage display peptide library technology. Intriguingly, its epitope is located in the E1E2 association site (WHY), encompassing CD81-binding region-1, as depicted on the predicted HCV E1E2 heterodimeric model proposed by Yagnik *et al.* (10) on the basis of tick-borne encephalitis virus. More exciting, such E1 and E2 sequences, which are recognized by our mAb D32.10, are also reactive with specific antibodies present in human sera from HCV-infected patients.

EXPERIMENTAL PROCEDURES

Antibody Production and Characterization—mAb D32.10 was generated as previously described.[2] Briefly, viral material was obtained in large amounts from plasmapheresis of a chronically HCV-infected patient (HCV-*L*, genotype 1b).

An HCV-enriched pellet was prepared by successive ultracentrifugations. The final pellet (concentrated 240-fold) contained ~10^7 copies of HCV RNA/mg of protein as determined by Amplicor™ HCV Monitor™ (Roche Diagnostics, Meylan, France). BALB/c mice were inoculated with 100 μg, *i.e.* 10^6 copies of HCV RNA (two injections), of this viral preparation and boosted with 50 μg (two injections) before fusion with X63 myeloma cells (11). Hybridoma culture supernatants were screened for the presence of HCV-specific antibodies by indirect enzyme immunoassay (EIA) and for HCV polypeptide specificity by immunoblotting using the immunogen as an antigenic probe (12). The HCV antigenic

* This work was supported by Agence Nationale de Recherche sur le SIDA (ANRS) Grant 2001/063 and by the "Réseau National des Hépatites." The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HCV, hepatitis C virus; mAb, monoclonal antibody; EIA, enzyme immunoassay; BSA, bovine serum albumin; ELISA, enzyme linked-immunosorbent assay; Endo H, endoglycosidase H; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

[2] Petit, M.-A., Jolivet-Reynaud, C., Peronnet, E., Michal, Y., Dubuisson, J., and Trépo, C., 11th International Symposium on Viral Hepatitis and Liver Disease Proceedings, April 6–9, 2003, Sydney, Australia.

reactivity recognized by mAb D32.10 was also analyzed by EIA of each fraction recovered after centrifugation in a sucrose density gradient of the HCV-enriched pellet.

HCV Indirect EIA—96-Well polystyrene plates (Falcon from BD Biosciences, Le Pont de Claix, France) were coated with four different HCV preparations (1 mg/ml protein) diluted from 10^{-1} to 10^{-6} (corresponding to 100 μg/ml to 1 ng/ml). The plates were incubated overnight at 4 °C and then saturated with 20 mM Tris-HCl (pH 7.5) and 100 mM NaCl containing 5% (w/v) bovine serum albumin (BSA) (Tris/NaCl/BSA buffer). mAb D32.10 diluted in a mixture of Tris/NaCl/BSA buffer and 50% normal human serum at a concentration of 5 μg/ml was added to each well and incubated for 2 h at 37 °C. The bound antibody was detected with a horseradish peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse immunoglobulins (diluted 1:5000; Immunotech) and with *o*-phenylenediamine and H_2O_2 as substrates. Absorbance was determined at 450 nm with an ELISA plate reader (MRX, Dynex). The results were considered as positive when superior to the cutoff, corresponding to the mean of negative controls multiplied by 2.1.

Western Immunoblot Experiments—The untreated HCV-enriched pellet (HCV-*L*) was used as an antigenic probe (12, 13) at concentrations from 0.1 to 1 mg/ml. The antigen was subjected to SDS-PAGE on 12.5% gels under reducing or nonreducing conditions (2% SDS ± 5% β-mercaptoethanol). After protein transfer onto polyvinylidene difluoride membranes, immunoblotting was performed using mAb D32.10 (2–5 μg/ml; diluted in 50% normal human serum) as the primary antibody. Mouse IgGs bound were then detected by incubation with a peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse immunoglobulins (diluted 1:10,000; Dako Corp.) as the secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL$^+$ system, Amersham Biosciences). Glycosidase digestion was performed as previously described by Sato *et al.* (14) on circulating HCV virions. The HCV-enriched pellet (HCV-*L*, 4 μg) was treated with 5, 10, or 20 milliunits/ml peptide *N*-glycosidase A (Roche Applied Science) in 100 mM citrate/phosphate buffer (pH 6.0) for 18 h at 37 °C. Deglycosylation of purified HCV particles was also performed by overnight incubation at 37 °C in 50 mM sodium acetate buffer (pH 5.5) containing endoglycosidase H (Endo H; 5 milliunits/μl; Roche Applied Science), 20 mM dithiothreitol, and 0.1% Triton X-100. The control digestion was performed under the same conditions as described for the peptide *N*-glycosidase A or Endo H digestion, except the enzyme was omitted. Samples were then treated with electrophoresis sample buffer containing reducing agent and analyzed by SDS-PAGE.

Dodecapeptide Library Screening—The Ph.D.-12™ phage display peptide library kit was obtained from New England Biolabs, Inc. This is a combinatorial peptide 12-mer fused to the minor coat protein (pIII) of M13 phage. The displayed peptide 12-mers are expressed at the N terminus of pIII. The library consists of ~1.9×10^9 electroporated sequences, amplified once to yield ~20 copies of each sequence in 10 μl of the supplied phage. Three biopannings were performed according to the manufacturer's instructions, with some modifications. Briefly, 10 μg of biotinylated mAb D32.10 were coupled to a 35-mm polystyrene Petri dish (Falcon) coated with 40 μg of streptavidin. The dish was incubated overnight at 4 °C and washed six times with 50 mM Tris and 150 mM NaCl (pH 7.5) containing 0.5% Tween 20 (TBS/Tween). In the first round of selection, 4×10^{10} phage from the initial library were allowed to react with the dish-bound IgG for 4 h at 4 °C under rocking conditions. The unbound phage were removed by repetitive washes with TBS/Tween. The bound phage were then eluted from the dish with 400 μl of elution buffer (0.1 N HCl (pH adjusted to 2.2 with glycine) and 1 mg/ml BSA). After neutralization with 75 μl of 1 M Tris-HCl (pH 9.1), the eluted phage were then amplified by infection of 20 ml of a 1:100 dilution of an overnight culture of *Escherichia coli* ER2537 (*recA*$^+$ strain cells) as recommended by the manufacturer. The culture was incubated for 4.5 h at 37 °C with vigorous shaking. Supernatants were obtained and precipitated with polyethylene glycol as previously described (15). In the second and third rounds of selection, 20% of the amplified phage from the preceding round were preincubated overnight at 4 °C with biotinylated mAb D32.10 at final concentrations of 10 and 1 nM, respectively, before being added to the 35-mm polystyrene Petri dish coated with 10 μg of streptavidin. The procedure was then identical to the first round. The phage from the third biopanning eluate were cloned and amplified for DNA sequencing and immunoanalysis.

DNA Sequencing—Single-stranded DNA was prepared from the purified phage as described by Sambrook *et al.* (16). The nucleotide sequence of the gene III inserts was determined according to the modified method of Sanger *et al.* (17) with an Applied Biosystems DNA sequencer (Model 377A) using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Life Sciences). Cycle sequencing was performed with primer 5′-HO-CCCTCATAGTTAGCGTAACG-OH-3′ corresponding to the pIII gene sequence. The amino acid sequence of the insert was deduced from the nucleotide sequence.

ELISA Using Supernatant Phage—Rows of ELISA plate wells were coated with 100 μl of either mAb D32.10 or an irrelevant mAb at a final concentration of 100 μg/ml in 0.1 M $NaHCO_3$ buffer (pH 8.6). The plates were incubated overnight at 4 °C and then blocked with 0.1 M $NaHCO_3$ buffer (pH 8.6) containing 5 mg/ml BSA. After 2 h of incubation at 4 °C, the plates were washed six times with TBS/Tween. 4-Fold serial dilutions of each phage clone were added to each well of the microtiter plate in a final volume of 100 μl of TBS/Tween, starting with 10^{12} virions in the first well of a row and ending with 2×10^5 virions in the twelfth well. The plates were incubated for 2 h at room temperature with agitation and then washed six times with TBS/Tween as described above. The bound phage were detected in a sandwich assay using horseradish peroxidase-conjugated anti-M13 monoclonal antibody at a 1:5000 dilution (Amersham Biosciences). The plates were developed using a commercial color kit (BioMérieux, Marcy l'Etoile, France) containing *o*-phenylenediamine and H_2O_2. After 10 min of incubation, the plates were read at 492 nm with an ELISA plate reader. For each phage clone dilution, the results are expressed as the difference between the value obtained with mAb D32.10 and the value obtained with the irrelevant monoclonal antibody. The results were then confirmed by testing optimal dilutions of the immunoreactive clones in triplicate.

Sequence Analysis—The amino acid sequences of peptides were compared with the HCV E1 and E2 protein sequences using MacVector Version 4.5 software (Eastman Kodak Co.). Basically, the regions of highest similarity were detected with the LFASTA program, which tentatively searches for best local identities (18).

Peptide Synthesis on Nitrocellulose Using Spot Technology—The simultaneous synthesis of different peptide sequences was performed on a nitrocellulose membrane using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acid chemistry (19). Each peptide was generated in nanomolar quantities suitable for immunological detection. Antibody reactivity with membrane-bound peptides was analyzed by an indirect colorimetric immunoassay as described previously (20). Spots corresponding to peptides with antibody reactivity produced a positive blue signal. Intensity of spots was estimated by visualization and expressed as relative intensity on a scale ranging from 0 to 5.

ELISA with Synthetic Peptides and Human Sera—Wells were coated overnight at 4 °C with 100 μl of streptavidin at a final concentration of 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) and blocked for 1 h at 37 °C with phosphate-buffered saline (PBS) containing 10% goat serum. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS/Tween) before adding 100 μl of biotinylated peptide solution (10 μg/ml in PBS) for 2 h at 37 °C. After another wash with PBS/Tween, 100 μl of serum diluted 1:50 in PBS/Tween containing 10% goat serum were added and incubated for 2 h at 37 °C. The plates were washed again with PBS/Tween. The peroxidase-conjugated goat anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was then added at a 1:5000 dilution in PBS/Tween containing 10% goat serum. The plates were incubated for 1 h at 37 °C and washed once more with PBS/Tween. The plates were developed using the commercial BioMérieux color kit containing *o*-phenylenediamine and H_2O_2. After 10 min of incubation, the plates were read at 492 nm with an ELISA plate reader. The values are the mean absorbance in triplicate.

RESULTS

mAb D32.10 Recognizes Native Serum-derived HCV1a and HCV1b Particles—Four different HCV RNA-positive enriched pellet preparations (HCV1, HCV2, HCV3, and HCV4) were obtained as previously described[2] from three different patients (*L.*, *F.*, and *Fan.*) and tested by EIA for their recognition by mAb D32.10. As shown in Fig. 1, mAb D32.10 detected up to 10 ng of protein/ml of each preparation. The detection was linear between 10 μg and 100 ng or 1 μg and 10 ng/ml protein. HCV1 (the immunogen, genotype 1b) and HCV2 were two preparations derived from the same patient (*L.*). HCV3 (*F.*) and HCV4 (*Fan.*) were obtained from two other patients with severe cutaneous vasculitis, cryoglobulinemia, and chronic hepatitis C requiring plasma exchanges. The patient *Fan.* (HCV4) was initially found to have two distinct genotypes in serum, 1a and 2a (21). Our results show that mAb D32.10. is able to recognize determinants not restricted to genotype 1b of the immunogen.

mAb D32.10 Reacts with Large E1E2 Complexes and E1 and

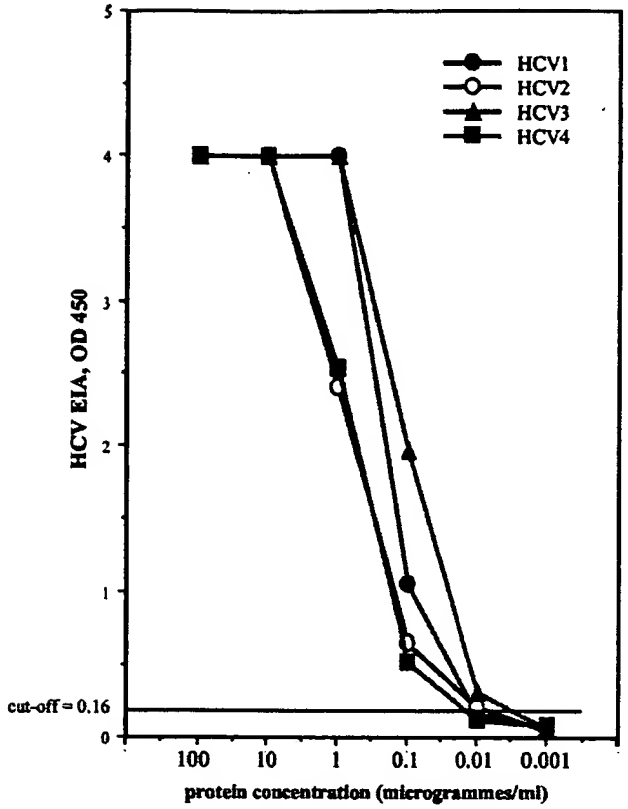

FIG. 1. **mAb D32.10 reactivity with HCV particles in EIA.** The binding of mAb D32.10 to HCV particles of genotype 1b (HCV1, HCV2, or HCV3) or 1a (HCV4) purified from three different chronically infected patients was measured by indirect EIA. Plates were coated with the individual HCV-enriched pellets used at a concentration of 1 mg/ml and diluted from 10^{-1} (100 μg/ml) to 10^{-6} (1 ng/ml). Purified mAb D32.10 IgGs were used at a concentration of 5 μg/ml and diluted in Tris/NaCl/BSA containing 50% normal human serum. The antibody bound to antigen-coated wells was then reacted with horseradish peroxidase-conjugated anti-mouse immunoglobulins as the secondary antibody; plates were revealed using *o*-phenylenediamine and H_2O_2; and the absorbance was read at 450 nm. The cutoff value corresponds to the mean of three negative controls multiplied by 2.1.

E2 Glycoproteins—Using the immunogen (HCV1) as an antigenic probe, the HCV polypeptide specificity of mAb D32.10 was tested by immunoblot analysis under reducing and nonreducing conditions (Fig. 2*A*). When two concentrations of the same sample (2.5 and 5 μg) (*lanes 2* and *3*, respectively) were analyzed under reducing conditions (2% SDS + 5% β-mercaptoethanol), mAb D32.10 recognized major bands at 60–68 kDa and another band at 31 kDa, corresponding to E2 and E1, respectively. mAb D32.10 also recognized disulfide-linked complexes recovered in the upper part of the gel (>200 kDa) when the HCV1 preparation was treated with 2% SDS only. These high molecular mass bands (*lane 1*) could correspond to hetero-oligomeric E1E2 complexes.[2]

Asparagine-linked complex-type sugar chains have been shown to be present on the surface of native virions of HCV (14); thus, the ability of mAb D32.10 to recognize HCV-specific proteins after treatment of the HCV1 preparation with peptide *N*-glycosidase A at different concentrations (20, 10, and 5 milliunits/ml) was examined. As shown in Fig. 2*B* (*lanes 1–3*, respectively), mAb D32.10 reacted with all of the deglycosylation products of E1 (28, 25, 23, and 21 kDa), but especially with the 25-kDa (twice glycosylated) and 21-kDa (non-glycosylated) species, which accumulated at the highest concentration of the enzyme. Although E1-related products could be clearly detected by mAb D32.10 after deglycosylation, neoformed E2-related products were not identified after the treatment. The sensitivity of E1E2 complexes expressed on natural HCV particles to Endo H digestion was investigated. As shown in Fig. 2*C*, only a diffuse shift in molecular mass was observed for both E2 (from 68 to 42 kDa) and E1 (from 34 to 24 kDa) proteins, suggesting that E1 and E2 on serum-derived native HCV particles possess mainly a complex Endo H-resistant glycosylation.

Epitope Mapping of mAb D32.10 Using Phage Display—To further characterize the epitope recognized by mAb D32.10, the antibody was used to screen a phage display dodecapeptide library. After three rounds of selection, 4% the phage input was found in the eluate, indicating amplification of specifically bound phage. Thus, 88 clones were randomly isolated; their DNAs were sequenced; and the amino acid sequences of inserts were deduced. Forty-eight different sequences were obtained, and some of them were found in several examples. However, when tested in an ELISA for their immunoreactivity with mAb D32.10, none of them gave a positive signal, indicating that the binding affinity was too low to be detectable. The 48 clone sequences were compared with the sequences of HCV E1 and E2. Five and three sequences presented similarities to residues of E1 located in regions 292–305 and 347–356, respectively (Table I) whereas seven, four, and two sequences shared some similarities with residues of E2 located in regions 481–501, 610–631, and 685–698, respectively (Table II). Moreover, as shown in Table III, three of these different motifs shared similarities with both E1-(292–305) and E2-(482–499) or E2-(612–626), and two of them shared similarities with both E1-(347–356) and E2-(482–499) or E2-(612–626).

Immunoreactivity of Selected Phage-displayed E1 and E2 Sequences with mAb D32.10—To evaluate the significance of these different localizations on both E1 and E2 sequences, regions 291–315 and 347–356 of E1 and regions 473–498, 607–627, and 686–697 of E2 were reproduced by the Spotscan approach as overlapping synthetic pentadecapeptides offset by one and tested for their immunoreactivity with mAb D32.10. A strong positive signal was obtained with peptides corresponding to E1-(292–306) (Table IV, part A), whereas E1-(347–356) was not recognized by mAb D32.10 (data not shown). As shown in Tables V (part A) and VI (part A), peptides corresponding to E2-(482–499) and E2-(612–626), respectively, were also immunoreactive with mAb D32.10, and no signal was detected with E2-(686–697) (data not shown). The immunoreactive E1 and E2 regions were reproduced as overlapping octapeptides. Indeed, the immunoreactivity of E1-(292–306) (pentadecapeptide TFSPRRHWTTQGCNC) could by restricted to ^{297}RHWTTQGCNC^{306} (Table IV, part B), and that of E2-(608–622) (pentadecapeptide LVDYPYRLWHYPCTI) to ^{613}YRL**WHYPC**T^{621} (Table VI, part B). However, as shown in Table V (part B), overlapping octapeptides corresponding to E2-(480–494) (pentadecapeptide PDQRPYC**WHYP**PKPC) gave two non-overlapping zones of weaker immunoreactivity: ^{479}GPDQRPYC^{486} and ^{487}WHYPPKPC^{494}, indicating that the recognition of each octapeptide by mAb D32.10 is partial. Indeed, it is difficult to discriminate between E2-(480–494) and E2-(613–621) because these two regions contain the same motif (**WHYP**) reported by Yagnik *et al.* (10) to be involved in the heterodimerization of E1E2.

Determination of Critical Residues of the mAb D32.10 Epitope by Alanine Replacement Analysis—The contribution to antibody binding of the conserved residues between the phage-displayed peptides and the E1 and E2 proteins was assessed by preparing a series of alanine analogs of sequences 292–306, 480–494, and 608–622. A residue was defined as critical to binding if its replacement with alanine induced a decrease of at least 50% in the signal of the corresponding peptide compared

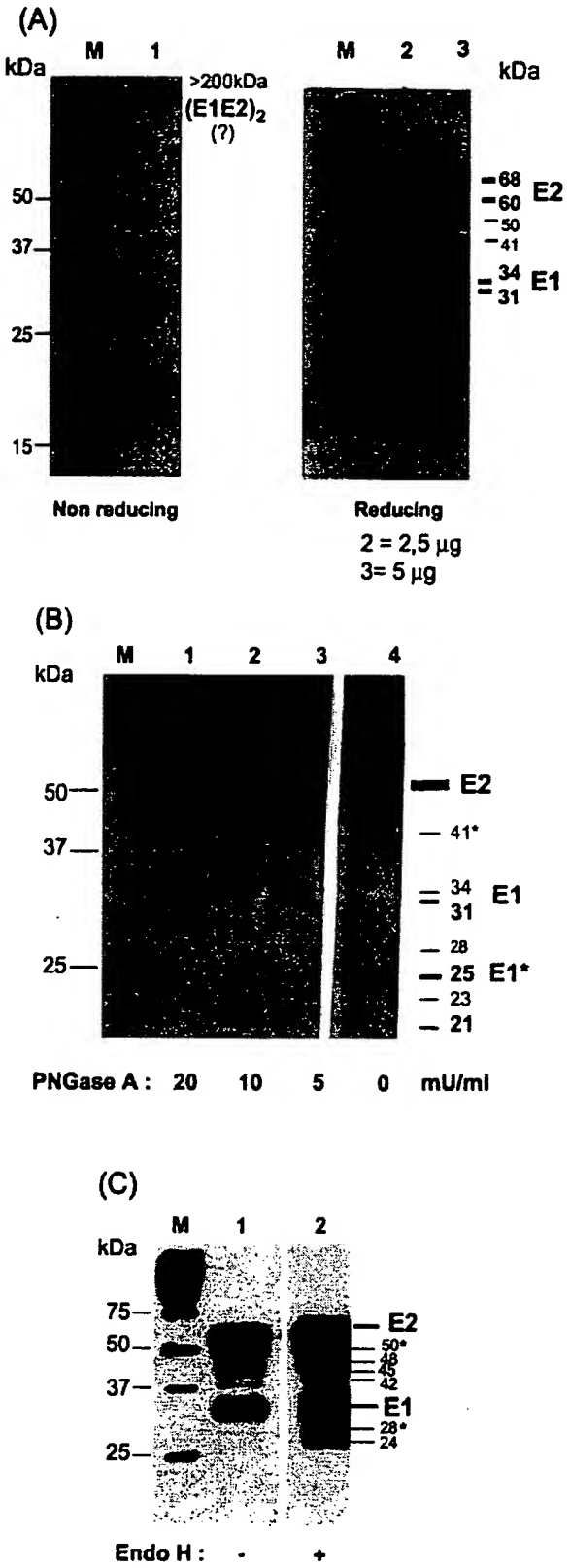

FIG. 2. **mAb D32.10 reactivity with HCV proteins in Western blotting.** *A*, serum-purified HCV particles (HCV1, genotype 1b) were used as antigenic probes to determine the polypeptide specificity of mAb D32.10 under nonreducing (*lane 1*) and reducing (*lanes 2* and *3*) conditions. *B*, the effect of peptide *N*-glycosidase A (*PNGase A*) at 20 (*lane 1*), 10 (*lane 2*), 5 (*lane 3*), and 0 (*lane 4*) milliunit/ml on the binding of mAb D32.10 to HCV envelope proteins E1 and E2 was studied. *C*, the HCV-enriched pellet was untreated (*lane 1*) or was treated with Endo H (5 milliunits/µl) (*lane 2*). The proteins were subjected to SDS-PAGE; and after transfer, immunoblotting was performed using mAb D32.10 diluted in 50% normal human serum to a final concentration of 3 µg/ml. Antibody binding was detected with horseradish peroxidase-conjugated anti-mouse immunoglobulins as the secondary antibody. Bands were visualized using the ECL* system. The molecular masses of markers (*lanes M*) and HCV proteins are indicated in kilodaltons on the *left* and *right*, respectively. *Asterisks* indicate deglycosylated forms of E1 and E2.

TABLE I
Amino acid similarities between selected phage clones and E1 sequences
Amino acid similarities to E1 are indicated in boldface.

E1(289-307)	QLFTFSPRRHWTTQGCNCS
Clone 1	**SP**L**R**H**Y**EL**P**LI**Q**
Clone 2	W**P**HN**HS**T**H**SR**TH**
Clone 3	FPK**YHPR**F**HK**H**A**
Clone 4	SQRS**RHW**HDVPK
Clone 5	**TSQPR**W**HQ**KPAT
E1(343-363)	AILDMIAGAHWGVLAGIAYFS
Clone 6	WK**M**PRA**T**D**WNL**R
Clone 7	**HWG**NHSKSHPQR
Clone 8	WHRTPS**TLWGVI**

TABLE II
Amino acid similarities between selected phage clones and E2 sequences
Amino acid similarities to E2 are indicated in boldface.

E2(481-501)	DQRPYCWHYPPKPCGIVPAKS
Clone 9	**WH**KL**P**GHPRTV
Clone 4	SQ**R**SRH**WH**DV**PK**
Clone 10	T**FAWH**K**P**RVNL**G**
Clone 11	TSQPR**WH**QK**P**AT
Clone 12	HSSW**Y**IQ**HFPP**L
Clone 13	**FPAHP**LPR**LPSL**
Clone 8	**WH**RT**P**STLWG**VI**
E2(610-631)	DYPYRLWHYPCTINYTIFKIRM
Clone 1	S**P**L**R**HYEL**P**LIQ
Clone 14	**WH**WNKP**I**R**PPLR**
Clone 15	Q**PYKL**QAAA**T**LY
Clone 6	**WK**M**PRAT**D**WNLR**
E2(685-698)	LSTGLIHLHQNIVD
Clone 16	H**LYHKNRN**HIAY
Clone 17	WS**P**GQQ**RLHN**ST

with the unmodified peptide. As shown in Fig. 3, for the three peptides, the only critical residues were Cys^{306}, Cys^{494}, and Cys^{620}, respectively, whereas the replacement of Cys^{304} and Cys^{486} with alanine in peptides 292–306 and 480–494, respectively, had no effect. Regarding the contribution to antibody binding of the conserved residues between the phage-displayed peptides and the E1 and E2 proteins, the absence of an effect of the corresponding alanine analogs on the respective immunoreactivities of the three peptides is in agreement with the fact that the peptides displayed on phage did not give a detectable

TABLE III
Amino acid similarities between phage clones and both E1 and E2 sequences

Amino acids in boldface correspond to similarities to E1, and underlined amino acids to similarities to E2.

E1-(292–305), E2-(482–499/612–626)	
Clone 1	**SPLRHYELPLIQ**
Clone 4	**SQRSRHWHDVPK**
Clone 11	**TSQPRWHQKPAT**
E1-(347–356), E2-(482–499/612–626)	
Clone 6	**WKMPRATDWNLR**
Clone 8	**WKMPRATDWNLR**

TABLE IV
Immunoreactivity of overlapping peptides offset by one spanning residues 291–315 of the HCV E1 glycoprotein

Shown is the reactivity of peptides with mAb D32.10 diluted to a final concentration of 20 μg/ml.

Peptides	E1-(291–315) sequence	Spot intensity
A. Pentadecapeptides	FTFSPRRHWTTQGCN	2
	TFSPRRHWTTQGCNC	4
	FSPRRHWTTQGCNCS	1.5
	SPRRHWTTQGCNCSI	2
	PRRHWTTQGCNCSIY	1
	RRHWTTQGCNCSIYP	0.5
	RHWTTQGCNCSIYPG	0
	HWTTQGCNCSIYPGH	0
	WTTQGCNCSIYPGHI	0
	TTQGCNCSIYPGHIT	0
	TQGCNCSIYPGHITG	0
B. Octapeptides	TFSPRRHW	0
	FSPRRHWT	0
	SPRRHWTT	0
	PRRHWTTQ	0
	RRHWTTQG	0
	RHWTTQGC	3
	HWTTQGCN	3
	WTTQGCNC	4
	TTQGCNCS	1

signal by themselves because they did not contain any Cys residue. Indeed, according to the manufacturer, the low frequency of Cys in the Ph.D.-12™ library (0.4% instead of 5–10% for the other amino acids) does not favor the selection of peptides with Cys. However, as this library provides an increased window of randomness, it allows the target to select sequences with multiple weak contacts instead of a few strong interactions. In conclusion, these results suggest that, in sequences 292–306, 480–494, and 608–622, Cys^{306}, Cys^{494}, and Cys^{620} are the only residues that react strongly with the antibody, whereas the other amino acids contribute equally and more weakly to the binding.

Immunoreactivity of the Selected E1 and E2 Sequences with Human Sera—E1-(292–306), E2-(480–494), and E2-(608–622), corresponding to the bound peptides that gave the greatest signals with mAb D32.10 in the Spotscan approach, were reproduced as biotinylated synthetic peptides. To validate the localization of the mAb D32.10 epitope, we assessed the availability of these sequences on the viral particle surface as well as their potential immunogenicity during *in vivo* HCV infection by testing these peptides with 44 sera from HCV-infected patients and 11 sera from healthy individuals. Using a cutoff of recognition calculated for each peptide (mean of the values obtained with HCV-negative sera + 3 S.D.), positive responses were obtained with 6 out of 44 HCV-positive sera against E1-(292–306) (Fig. 4*A*), 6 out of 44 against HCV-positive sera E2-(480–494) (Fig. 4*B*), and 16 out of 44 HCV-positive sera against E2-(608–622) (Fig. 4*C*). Sera A7, A14, A21, A33, A39, and A40 gave a positive signal with the three peptides, whereas E2-(608–622) was also recognized by 10 more sera. This indicates that these E1E2 sequences or, more likely, the corresponding epitope recognized by mAb D32.10 was able to induce an immune response in some HCV patients during infection.

TABLE V
Immunoreactivity of overlapping peptides offset by one spanning residues 473–498 of the HCV E2 glycoprotein

Shown is the reactivity of peptides with mAb D32.10 diluted to a final concentration of 20 μg/ml.

Peptides	E2-(473–498) sequence	Spot intensity
A. Pentadecapeptides	SYANGSGPDQRPYCW	0
	YANGSGPDQRPYCWH	0
	ANGSGPDQRPYCWHY	0
	NGSGPDQRPYCWHYP	0
	GSGPDQRPYCWHYPP	0
	SGPDQRPYCWHYPPK	0
	GPDQRPYCWHYPPKP	0
	PDQRPYCWHYPPKPC	3
	DQRPYCWHYPPKPCG	0.5
	QRPYCWHYPPKPCGI	1
	RPYCWHYPPKPCGIV	1
	PYCWHYPPKPCGIVP	0.5
B. Octapeptides	SGPDQRPY	0
	GPDQRPYC	2
	PDQRPYCW	0
	DQRPYCWH	0
	QRPYCWHY	0
	RPYCWHYP	0
	PYCWHYPP	0
	YCWHYPPK	0
	CWHYPPKP	0
	WHYPPKPC	2
	HYPPKPCG	1
	YPPKPCGI	1
	PPKPCGIV	0

TABLE VI
Immunoreactivity of overlapping peptides offset by one spanning residues 607–627 of the HCV E2 glycoprotein

Shown is the reactivity of peptides with mAb D32.10 diluted to a final concentration of 20 μg/ml.

Peptides	E2-(607–627) sequence	Spot intensity
A. Pentadecapeptides	CLVDYPYRLWHYPCT	3
	LVDYPYRLWHYPCTI	4
	VDYPYRLWHYPCTIN	2
	DYPYRLWHYPCTINY	1
	YPYRLWHYPCTINYT	0
	PYRLWHYPCTINYTI	0
	YRLWHYPCTINYTIF	0
B. Octapeptides	LVDYPYRL	0
	VDYPYRLW	0
	DYPYRLWH	0
	YPYRLWHY	0
	PYRLWHYP	0
	YRLWHYPC	4
	RLWHYPCT	3.5
	LWHYPCTI	1
	WHYPCTIN	0

DISCUSSION

Until now, the low levels of HCV particles present in patient plasma and the lack of an efficient cell culture system for HCV propagation had precluded a direct analysis of the virion envelope glycoproteins. The envelope glycoproteins have been shown to assemble into a noncovalent E1E2 heterodimer that is retained in the endoplasmic reticulum (7). This heterodimer is believed to be the prebudding form of an HCV glycoprotein complex (8), which had been proposed as a functional subunit of HCV virions (9, 22). However, anti-HCV monoclonal antibodies (A4, A11, and H2) obtained from E1E2 heterodimers produced in such a heterologous system failed to bind to HCV RNA-containing particles from serum (7). This suggests that the

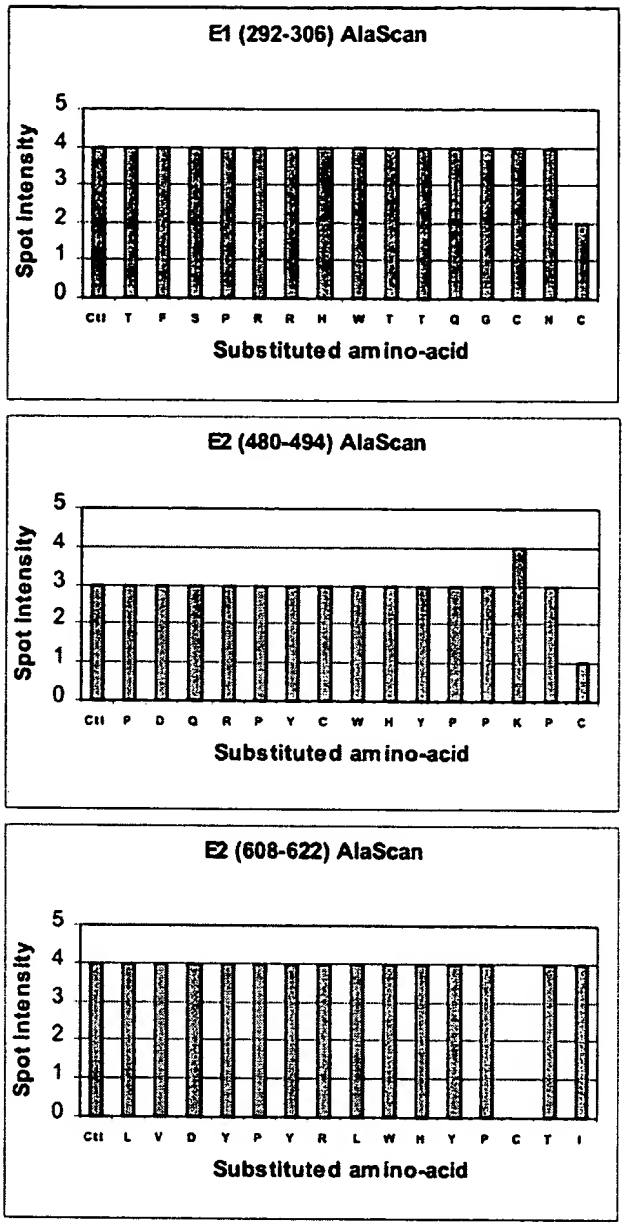

FIG. 3. **Analysis of critical residues of the mAb D32.10 epitope by alanine scanning.** Spot intensities correspond to the respective reactivities of the control and substituted peptides with mAb D32.10 diluted to a final concentration of 20 μg/ml.

exact nature of the interaction between the two HCV envelope glycoproteins, E1 and E2, is not yet known. Therefore, we produced monoclonal antibodies by immunization of mice with human serum-derived viral particles[2] to obtain a better knowledge of the three-dimensional structure of E1E2 complexes expressed on the surface of native HCV. Here, one particularly reactive monoclonal antibody (mAb D32.10) was selected for further characterization.

Our analysis of mAb D32.10 binding first established its specificity for HCV particles from different origins (subtypes 1a and 1b) with a relative high affinity as determined by limiting dilution in indirect EIA (reactivity up to 1 ng/ml protein for each preparation). Using denatured HCV proteins as antigenic probes in Western blotting, we determined that mAb D32.10 strongly reacted with a doublet at 60–68 kDa and more faintly with a doublet at 31–34 kDa, showing that mAb D32.10 recognizes the E2 envelope protein likely at different states of glycosylation and the E1 glycoprotein after reducing treatment of viral particles. There are many *N*-linked glycosylation sites within the amino acid sequences of E1 (up to 6) and E2 (up to 11) in all of the known HCV isolates of genotypes 1–4 (23). Furthermore, *in vitro* expression studies have indicated that the E1 and E2 gene products are associated with sugar chains when expressed in mammalian or insect cells (24, 25). Under nonreducing conditions, mAb D32.10 reacted with covalently associated complexes of ~200 kDa. Because mAb D32.10 bound both E1 and E2 and because the mAb D32.10 epitope was suspected to be involved in a nonlinear composite epitope, the method of phage display epitope mapping was chosen to identify residues of HCV proteins involved in mAb D32.10 binding. The phage-displayed sequences showed similarities to both E1 and E2 regions. Region 292–306 of E1 and regions 480–494 and 608–622 of E2 as pentadecapeptides interacted with mAb D32.10 in ELISA. Using overlapping octapeptides, we determined that ^{297}RHWTTQG**C**N**C**^{306} of the HCV E1 protein and both ^{613}YRL**WHYPC**T^{621} and ^{480}PDQRPY**C**W**HYP**PKP**C**^{494} of the HCV E2 protein were reactive with mAb D32.10. The two regions identified in E2 contained the same motif (**WHY**) reported by Yagnik *et al.* (10) as likely to be involved in the heterodimerization of E1E2. Indeed, it is difficult to discriminate between these two regions. Because two non-overlapping zones (^{479}GPDQRPY**C**^{486} and ^{487}**WHYP**PKP**C**^{494}) separately bound to mAb D32.10, this suggests that mAb D32.10 specifically recognized each octapeptide and thus the complete sequence (amino acids 480–494). The three identified regions contain cysteine (*C*) residues, which could be involved in intramolecular and/or intermolecular disulfide bridges. Indeed, Cys^{306}, Cys^{494}, and Cys^{620}, located in the C-terminal part of each peptide, were shown by alanine substitution to be the critical residues for strong reactivity with mAb D32.10. Interestingly, Cys^{620} from one monomer has been proposed as the most likely candidate for covalent interaction in E2 homodimerization (10). In addition, E2-(476–494), containing Cys^{486} and Cys^{494} and including the WHY motif, might be important for heterodimeric association between E1 and E2 (10). Finally, E1-(297–306), reacting with mAb D32.10, contains two amino acids (**NC**) in the C-terminal part belonging to the fourth core glycosylation site, Asn^{305}-X(Cys^{306})-Ser^{307}, which is involved in the coprecipitation of E1 and E2 and would be the less efficiently glycosylated (only 66% of E1) *in vitro* (26). On the other hand, it has been demonstrated that treatment of HCV particles recovered from the circulation of infected humans with peptide *N*-glycosidase A results in a significant decrease in their ability to bind to lectins RCAI (*Ricinus communis*) agglutinin-I and WGA (*Triticum vulgaris*) wheat germ agglutinin (14). Our data show that treatment of the HCV1 preparation with peptide *N*-glycosidase A led to deglycosylation of E1 and that mAb D32.10 recognized the deglycosylated forms, especially the 25-kDa species. This suggests that site 4 is very likely less efficiently glycosylated *in vivo* than *in vitro* (26, 27) and probably plays an important role in the protein folding and antigenicity of native HCV envelope complexes. Surprisingly, deglycosylation of serum-derived HCV particles with Endo H allowed us to only partially remove high mannose-type oligosaccharides (even at the highest concentrations under denaturing conditions) (data not shown), suggesting the presence of a mixture of both Endo H-resistant complex glycans and Endo H-sensitive forms. Thus, our data show that the E1 and E2 proteins in the natural HCV particles contain complex glycans that are susceptible to cleavage by peptide *N*-glycosidase A, but resistant to Endo H, indicating that circulating HCV particles pass through the Golgi during cellular transport.

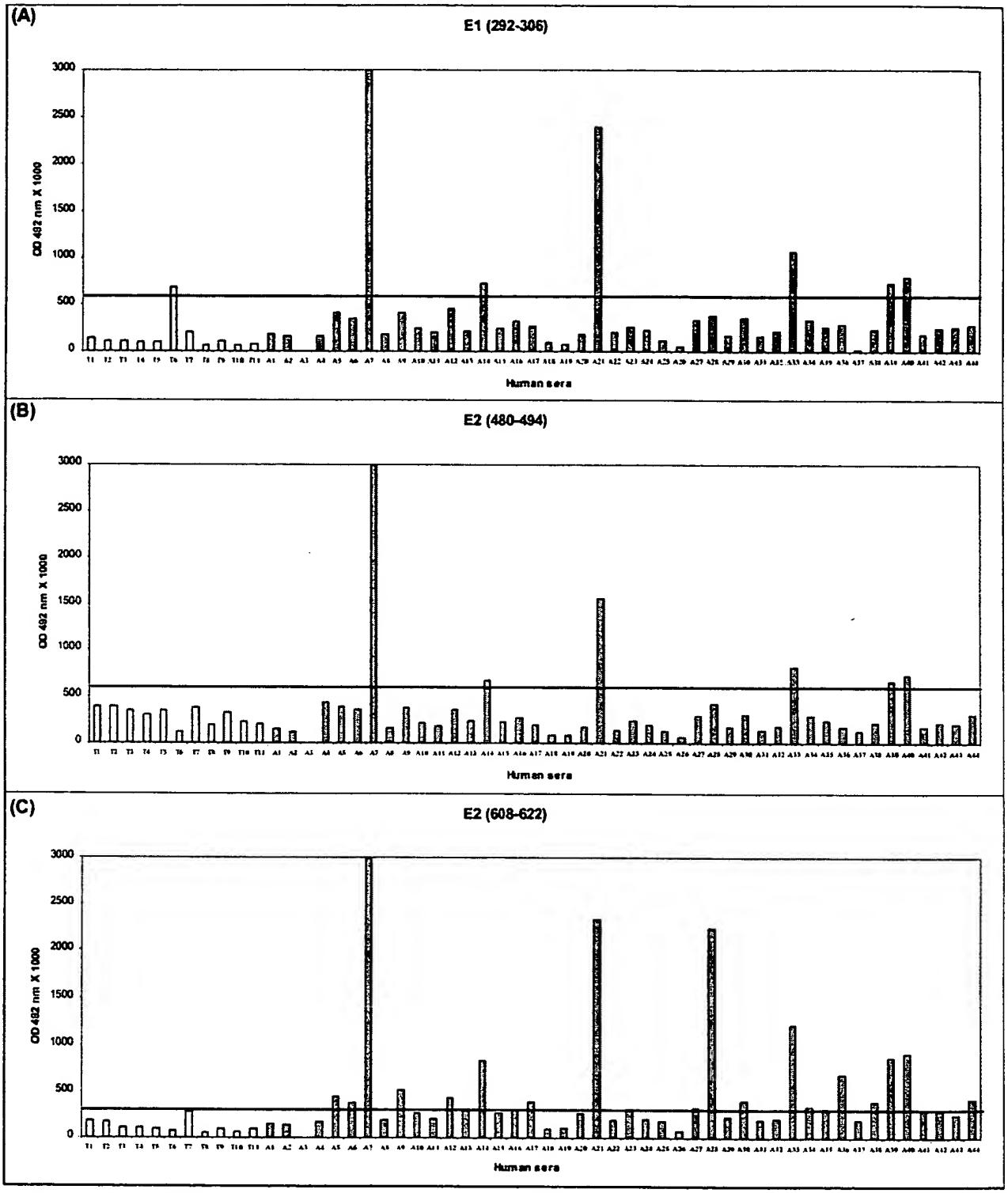

FIG. 4. **Human anti-HCV antibody binding to selected E1 and E2 sequences.** The immunoreactivity of biotinylated peptides was determined with sera diluted to 1:50. The T and A series correspond to sera from healthy individuals and from HCV-infected patients, respectively. The different recognition cutoff values, calculated as described under "Results," are as follows: 0.160 + (0.177 × 3) = 0.691 for E1-(292–306), 0.294 + (0.094 × 3) = 0.575 for E2-(480–494), and 0.121 + (0.067 × 3) = 0.321 for E2-(608–622).

Together, these results indicate that the three regions identified in E1 and E2 by mAb D32.10 are closely juxtaposed on the surface of serum-derived HCV particles. Secondary structure prediction of the ectodomain of HCV E2 using only HCV sequences suggested an overall low secondary structure content (≈37%) of predominantly β-strands. It is noteworthy that *Flavivirus* envelope glycoprotein E from tick-borne encephalitis virus shows functional similarity to HCV E2 (28), and these proteins are similar from the point of view of the parameters in these fold recognition methods (10). Tick-borne encephalitis virus envelope glycoprotein E could thus be considered as a good candidate for model building of HCV E2. In the current

model of HCV E2 (strain H), the 661-amino acid truncated E2 protein is sufficient to bind CD81 (29), to be exported (30), and to heterodimerize with E1 (31). Therefore, it could be assumed that amino acids 384–661 may represent the structural core of a functional E2 protein. All of these considerations and the puzzle specificity of our anti-E1E2 mAb D32.10 support the model proposed for the quaternary structure of envelope glycoproteins E1 and E2 by Yagnik *et al.* (10). Our results suggest that the mAb D32.10 epitope encompasses the E1E2 association site (amino acids 487–489, **WHY**), hypervariable region 2 (amino acids 474–482), and CD81-binding region-1 (amino acids 474–494) as well as residues 612–620, involved in CD81 binding and/or dimerization of E2. This implies that all of these regions would share the same site on the viral surface, in accordance with the formation of a head-to-tail E2 homodimeric pair, covalently linked, of heterodimers with E1 (10). Because such a model of HCV E2 was based on the tick-borne encephalitis virus glycoprotein E structure, it would be reasonable to assume that its physical relationship to the viral membrane could be also similar.[3] The presence in sera from HCV-infected patients of specific antibodies that were also able to react simultaneously with the three regions of E1 and E2 recognized by mAb D32.10 strongly supports their juxtaposition on the surface of circulating enveloped HCV particles and their immunogenicity in mice as well as in humans.

When baculovirus-derived HCV-like particles were used as a capture antigen in ELISA (32), no binding of mAb D32.10 was observed (data not shown), suggesting that envelope domains comprising amino acids 297–306 (E1), amino acids 480–494 (E2), and amino acids 613–621 (E2) are not accessible to this monoclonal antibody on the surface of HCV-like particles synthesized in insect cells. This finding may indicate that such recombinant particles (33) express envelope proteins in a conformation presenting antigenic properties that are distinct from those of the HCV particles circulating in the sera of chronically infected patients. The different patterns of envelope expression either in insect cells (two glycosylated forms of E1) or in mammalian cells (a single glycosylated protein) involve significant differences in glycosylation, which could probably explain the differences in the particles' morphology and antigenicity.

The HCV envelope glycoprotein complex is likely a key antigenic structure for an effective vaccine against the virus. Knowledge of its exact structure on the surface of native HCV particles will therefore present a significant step forward. A monoclonal antibody such as D32.10 that recognizes potential E2E2 and E1E2 association sites and the CD81-binding site could then become a valuable tool for studying HCV neutralization mechanisms and for identifying complete enveloped infectious HCV virions needed for further structural analysis of the native HCV envelope E1E2 complexes.

Acknowledgments—We gratefully thank Sandrine Busseret-Michel and Marjory Lièvre for data processing.

[3] M.-A. Petit, Y. Michal, S. Peyrol, and C. Trépo, manuscript in preparation.

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